Intracerebroventricular Injection of Rats: A Sensitive Assay Method for Endogenous Pyrogen Circulating in Rats (41015)[1]

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Abstract. Intracerebroventricular (icv) injection of endogenous pyrogen (EP) into rats causes dose-related fever. To compare this procedure with the usual assay method, iv injection into rabbits, a preparation of crude rabbit EP was titrated by both methods. The rat icv injection procedure gave results similar in linearity and magnitude of response to the conventional rabbit iv method, but was found to be at least 50-fold more sensitive. The icv assay worked for human as well as rabbit EP and for partially purified as well as crude preparations. Plasma from human or animal sources did not interfere. The rat icv and rabbit iv assay methods were also compared in their ability to detect a pyrogen in filter-sterilized plasma from rats infected with the live vaccine strain (LVS) of *Francisella tularensis*. The rat icv method gave fevers of 1° with 0.05-ml samples of the filtered plasma from infected rats, but no fever with plasma from control rats. The pyrogen was partially purified from the infected rat plasma. It was found to be heat labile and nondialyzable, and was tentatively identified as rat EP. The less sensitive rabbit assay was unable to detect the plasma pyrogen, using 0.05- and 1.00-ml samples. These observations suggest that icv injection into rats is a useful and sensitive assay method for EP, particularly when only small volumes are available for testing.

Fever in infected mammals has been attributed to the release of endogenous pyrogen (EP) into the bloodstream and its subsequent action upon the hypothalamic region of the brain (1). EP affects temperature-regulating neurons, resulting in an alteration of the "set-point" for body temperature (2). Endogenous pyrogen is a small (15,000 dalton) heat-labile protein produced upon stimulation of phagocytic cells (3).

Its biological activity is currently detected by two methods: a well-established rabbit bioassay (4) and a newly introduced, sensitive mouse bioassay (5). The much

greater sensitivity observed with the mouse assay compared to the rabbit method is offset by the fact that it is not quantitative except for a narrow range of EP concentrations. This is unfortunate because the more sensitive the assay, the lower the concentration at which the pyrogen can be detected. Furthermore, less material is used per assay, so that EP supplies can be conserved. Intracerebroventricular (icv) administration of small volumes of crude rabbit EP has been shown (6) to result in hyperthermia in rats. An attempt was therefore undertaken to quantitate this effect and determine its usefulness for measuring EP. The results reported here indicate that icv injection in rats is a sensitive, quantitative assay method for EP activity with distinct advantages over both mouse and rabbit bioassays.

Materials and Methods. Cannulas, guide cannulas, dummy inserts, cranioplastic cement, and mounting screws were obtained from Plastic Products Company, Roanoke, Virginia. Guide cannulas were implanted into male Fisher–Dunning rats (200–225 g) as described by Bailey *et al.* (6). Injections were performed using sterile, pyrogen-free, 1-ml plastic syringes, nee-

dles, and polyethylene tubing. The plastic syringe was mounted in a "stepper" automatic dispenser (PGC Scientific Corp., Rockville, Md.). Aliquots of 20–50 μl of samples containing EP or heat-inactivated EP (100°, 10 min) were injected through the cannula.

Rectal temperatures were recorded with a 700-series thermistor probe (Yellow Springs Instrument Co., Yellow Springs, Ohio), inserted 6.5 cm into the rectum, and a Model 5650 digital thermometer (Markson Science Inc., Del Mar, Calif.).

The rabbits used for bioassay were acclimated and treated as described by Mapes and Sobocinski (7). Only those which exhibited stable baseline temperatures and showed no febrile response to iv injection of 1 ml physiological saline were used for these studies.

Crude rabbit pyrogen was prepared from glycogen-induced peritoneal exudates (1 × 10^8 cells/ml) (8). Human EP was prepared from cultured U937 cells, as described by Bodel et al. (9). Endotoxin was measured by the Limulus amebocyte lysate (LAL) procedure, using a kit from Microbiological Associates, Walkersville, Maryland. Gel filtrations were done using a 2.5 × 40-cm column of Bio-Gel P-60 with normal saline buffered at pH 7.4 with 0.01 M sodium phosphate as the eluant and equilibration buffer.

In the study with infected rats, 10^7 colony-forming units of the live vaccine strain (LVS) of Francisella tularensis/100 g body wt. were injected ip into male Fisher–Dunning rats. Control rats received heat-inactivated LVS F. tularensis. The heat inactivation was performed at 56° for 1 hr, and the bacteria viability was checked by culture. The control rats displayed no fever or other symptoms of illness following inoculation. Details on the growth and culture of this bacterial strain were described previously (10, 11). Heparinized blood was collected 24 hr after the injection by anesthetizing the rats lightly with halothane, opening the thoracic and abdominal cavities, and removing 1–2 ml of blood from the heart using a needle and syringe containing approximately 20 units heparin. The blood was then centrifuged at 10,000g for 1 min with a Beckman microfuge, and the plasma was removed with a syringe and passed through a 0.22-μm filter.

Results. Intracerebroventricular injection of 50 μl of crude rabbit pyrogen from peritoneal exudates into rats results in significant fever by 2 hr, as indicated in the typical results shown in Fig. 1. Control rats injected icv with 50 μl of either saline or heat-inactivated rabbit pyrogen showed a similar lack of response.

Figure 2 shows results of the rat icv assay system compared with results for the rabbit iv assay. Both groups of assay animals received dilutions of the same preparation of rabbit EP. The data for the rabbit model are in good agreement with literature values
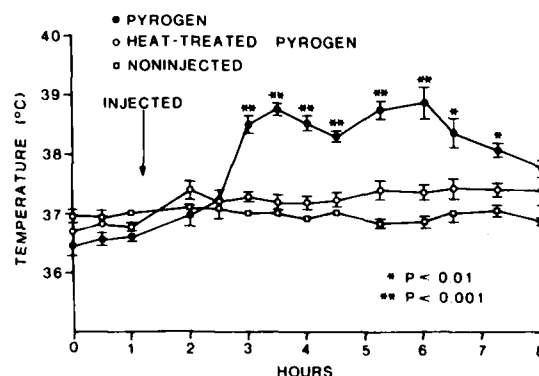

FIG. 1. Temperature response of rats to EP. The mean rectal temperatures ± SEM are plotted for groups of four rats injected icv with 50 μl of the samples indicated.
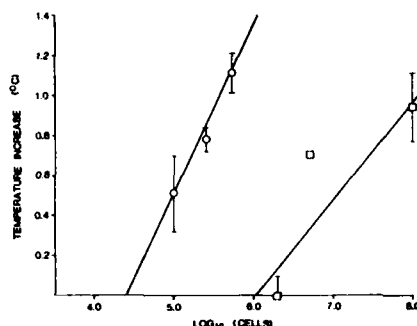
Fig. 2. Comparison of EP assay procedures. The increase in temperature caused by iv injection of EP into rabbits (□) or icv injection into rats (●) is plotted against the number of cells which produced the EP dose injected. Both models used EP from the same preparation of glycogen-stimulated rabbit peritoneal exudate cells. The data are mean ± SEM, $n = 5$, for the rat study and $n = 2$ for the rabbit study.

(12, 13). Individual temperature determinations for each rat and rabbit were examined by analysis of variance, and the elevations of the two assay lines were found to differ significantly ($P < 0.001$). As can be seen, approximately 100-fold less EP is required for a 1° rise in temperature with the rat assay system than the rabbit system. Table I shows that human as well as rabbit EP can be measured by the icv assay system, in both crude and partially purified states. The partial purification was by gel filtration; the fractions, combined and reconcentrated for these studies (6000–20,000 daltons), were chosen to include the EP monomer (15,000 daltons) but not prostaglandins or other low-molecular-weight species. Therefore, since the fevers in-

duced by the EP preparations before and after gel filtration are comparable, prostaglandins are not responsible for the observed fevers. Heating the EP preparations removed their pyrogenicity. Taken together, the molecular weight range and the heat lability of the EP fractions pooled argue against endotoxin as the cause of the fever in the assay, and in fact, the endotoxin concentration was found to be below the 1 ng/ml detection limit of the Limulus test.

As indicated in the table, human plasma itself did not cause fever in the assay system. Plasma or serum from other sources, such as goat, rabbit, and fetal calf, also produced no fever.

The reproducibility of the icv assay on cannulated rats utilized four times over a 1-month period is shown in Fig. 3. In this experiment, three dilutions from a preparation of crude rabbit EP were made and stored frozen between uses. Following cannula implantation and a 10-day recovery period, crude EP was injected icv into the rats. Fifty-microliter portions of each dilution of EP were administered in quadruplicate, and rectal tenperatures were recorded hourly. A group of four control rats were injected icv with 50 μl of heat-inactivated EP. This was repeated on each of the following 3 weeks. The data plotted are the increases in temperature of the rats receiving rabbit EP over those of the control rats.

A 7-day period was allowed between injections because it was found that injections on successive days, even of saline, caused elevated temperatures during the second group of assays. The guide cannulas become less firmly mounted in the skull ap-

TABLE 1. COMPARISON OF RABBIT AND HUMAN EP PREPARATIONS

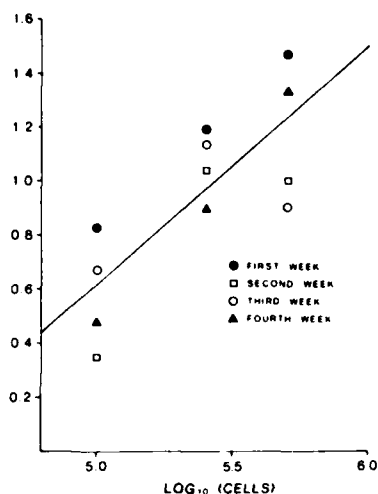| Sample	Purification	Concentration (cell equivalents/dose)	Temperature change ± SEM ($n = 3$)
Rabbit EP	Crude	5 × 10⁶	1.57 ± 0.35
	Gel filtered	5 × 10⁶	1.07 ± 0.16
	Crude, heated	5 × 10⁶	0.24 ± 0.22
Human EP	Crude	2 × 10⁶	1.90 ± 0.16
	Gel filtered	2 × 10⁶	1.65 ± 0.33
	Crude, heated	2 × 10⁶	0.42 ± 0.22
Human plasma	Crude	NA	0.29 ± 0.31

Fig. 3. Repeatability of icv assays. The increase in mean rectal temperature of groups of four rats injected icv with three dilutions of EP above the temperature of a group receiving heat-inactivated EP is plotted as a function of the number of cells which produced the 50-μl EP dose injected. The standard error on each point is ±0.2°.

Fic. 4. Assay for circulating EP. Mean rectal temperature ± SEM of a group of 10 rats receiving 50 μl icv of plasma from LVS *F. tularensis*-infected rats (■) and a group of 10 rats receiving 50 μl plasma from control rats injected with heat-killed LVS *F. tularensis* (△) are plotted as a function of time. The plasma was taken 24 hr after the rats were injected. The significance vs control values was determined by Student's paired *t* test.

proximately 6 weeks following implantation and the rats must be killed at that time.

An experiment was performed with plasma from LVS *F. tularensis*-infected rats to determine if the icv assay is sensitive enough to detect circulating EP. LVS *F. tularensis* infection in rats has been well described (10, 11). With a 10^7 bacteria/100 g body wt dose injected, a sustained fever began at 12 hr after inoculation, reaching 1° at 24 hr and 2° at 48 hr. This dose is 10% fatal at 36 hr, and the infected rats rarely survive to 72 hr. The control rats receiving heat-killed *F. tularensis* did not develop fevers or become ill.

Samples (50 μl) of filter-sterilized plasma from infected and control rats were injected into two groups of 10 cannulated rats. As shown in Fig. 4, the plasma from the infected rats produced a significantly greater fever than did that of the control rats. The plasma was shown not to contain endotoxin by the icv bioassay, since the pyrogen was found to be heat labile, and to give rise to a fever pattern typical of EP rather than endotoxin. Any endotoxin present in the
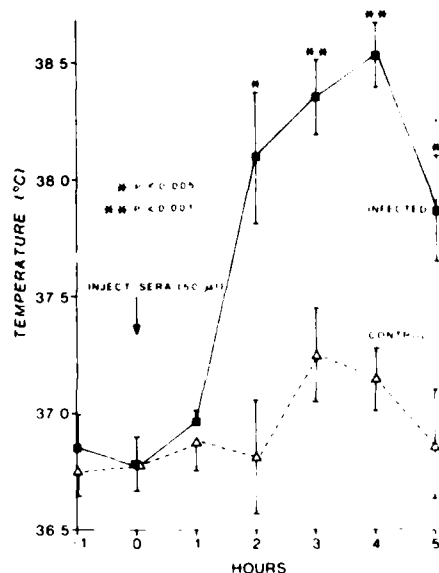
plasma was below the 1 ng/ml detection limit of the Limulus amebocyte lysate test.

Such a trace amount of endotoxin is not detectable by the icv bioassay. For example, a sample of rabbit EP found to contain endotoxin at a concentration of about 5 ng/ml by the Limulus test gave a fever of 1.7° (±0.3 SD) 2 hours after icv injection into rats, of which the endotoxin contribution was found to be 0.37° (±0.15), by comparison with heat-treated controls.

As previously reported (15), filter-sterilized plasma from this group of infected rats has been partially purified by affinity chromatography and dialyzed, with retention of all its pyrogenicity. This argues against the possibility that serum constituents other than EP, especially prostaglandins and other small molecules, are responsible for the fevers induced by the plasma. Serum samples that had been fro-

zen for 6 months before assaying gave results similar to those shown in Fig. 4 for fresh serum (data not shown).

Table 2 shows the results of experiments comparing the rat icv bioassay with the rabbit iv bioassay in measuring the rat plasma pyrogen. Assay rats receiving 50 μl of filter-sterilized plasma from infected rats subsequently had rectal temperatures more than 1° higher than those of control assay rats receiving identically treated control plasma, or heated plasma from infected rats. A 50-μl dose of the filter-sterilized plasma from the infected and control rats injected iv into assay rabbits produced no temperature change over the subsequent 3 hr. The dose was then increased 20-fold by injection of 1 ml of the plasma samples, with the plasma from infected rats causing a temperature rise of only 0.1 to 0.4° above the control. To show that these rabbits were capable of giving a positive response to endogenous pyrogen, 10^8 cell equivalents of crude rabbit EP were administered iv 3 days later, and found to produce 1° of fever in the assay rabbits.

Discussion. The previously reported information that icv injection of small quantities of rabbit enodgenous pyrogen into rats causes hyperthermia (6) was confirmed in this study and extended to show that it can be useful as a sensitive assay method for EP.

The experiments dealt with human and rabbit EP preparations and with a pyrogen in the plasma of *F. tularensis*-infected rats.

Evidence that the fever-inducing material used in these experiments was EP and not endotoxin includes its heat lability, the appearance of fever at 2 hr rather than at the 4 hr expected for endotoxin, and the fact that endotoxin was not detected in any of the samples by the Limulus amebocyte lysate assay. Testing of gel-filtered EP preparations indicated that the substance the icv assay was measuring had the approximate 15,000-dalton molecular weight expected for EP. Partial purification of the pyrogenic material in the rat plasma used in these experiments has been reported (15). The evidence indicates that the rat plasma pyrogen binds affinity material in the same manner as EP from human and rabbit sources do, and that it is not removed by dialysis. Because of the gel filtration and dialysis evidence, prostaglandins and other low-molecular-weight substances in either the pyrogen preparations or the plasma are unlikely to be responsible for the observed fevers.

The results show that the icv assay was nearly two orders of magnitude more sensitive than the conventional assay procedure; i.e., iv injection of rabbits. The mouse iv assay method is also reported (5) to be very sensitive, measuring 10^4 cell equivalents of EP. Unfortunately, it has the drawback of being restricted both in the magnitude of the temperature changes generated (0–0.5°) in response to EP and in the range of concentrations over which the assay is functional.

TABLE II. Comparison of Temperature Changes Induced by Plasma in Rat and Rabbit Assays

Plasma sample (n)	Volume injected (ml)	Temperature change (mean °C ± SEM)
Rat Assay		
Infected (4)	0.05	1.65 ± 0.17
Control (4)	0.05	0.30 ± 0.20
Heated (4)	0.05	0.29 ± 0.12
Rabbit Assay		
Infected (2)	0.05	−0.24, −0.48
Control (1)	0.05	−0.11
Infected (2)	1.00	0.29, 0.61
Control (1)	1.00	0.22
Rabbit EP (10^8 cell		
equivalents) (3)	1.00	1.10 ± 0.17

The cannulated rats have been used four times over a 1-month period following recovery from surgery, with good reproducibility of fever response. They can be kept in individual cages under normal animal laboratory conditions, in contrast to the 35° incubators required for assays with the mouse iv procedure (5). The amount of space required was, of course, much less than that for an equal-sized rabbit colony.

The sensitivity of the rat icv assay has allowed the detection of a pyrogen in the serum of *F. tularensis*-infected rats at levels far less than could be seen with the rabbit assay. A 1.65° fever was induced in the rat assay system by 0.05 ml of the plasma. Neither 0.05 or 1.00 ml produced significant fever when the same sample was used in the conventional rabbit assay. Results with different infections reported in the literature (11, 14, 16) indicate that 10 ml is normally injected for the observation of EP in serum samples using the rabbit assay.

Initial steps were taken which suggest that this assay method could be extended to clinical samples; human EP was found to be measurable by this method and human plasma did not interfere with the results. Goat plasma, rabbit serum, and fetal calf serum were also tested and found not to interfere with the assay.

A radioimmunoassay (RIA) for human EP has been developed (13) which provides a sensitive means of measurement, although it remains to be demonstrated that it can detect circulating EP. This assay, which is a solid-phase method, detected EP in concentrations as low as 0.01 to 0.1 rabbit pyrogen doses A rabbit pyrogen dose is defined as a rise of 0.6–0.9° in rectal temperature following iv injection. A similarly defined icv rat pyrogen dose would be at least 50 times less than a rabbit pyrogen dose. A 0.01 rabbit dose should therefore cause a fever of 0.3° or more in rats by the icv method. Thus, the lower limits of both the published solid-phase RIA and the rat icv assay were similar. It is probable that

more refined RIA methods can detect much lower EP concentrations. However, because of the difficulties and cost of preparing reagents for the RIA, and especially considering the lability of the EP itself, the rat icv model remains a viable alternative EP assay procedure. This is especially true if the experimenter wants to be sure the material he is testing is biologically active pyrogen.

1. Cooper, K. E., Cranston, W. I., and Honour, A. J., J. Physiol. (Lond.) 191, 325 (1967).
2. Cranston, W. I., Brit. Med. J. 2, 69 (1966).
3. Bodel, P., Wechsler, A., and Atkins, E., Yale J. Biol. Med. 41, 376 (1969).
4. Root, R., Nordlund, J. J., and Wolff, S. M., J. Lab. Clin. Med. 75, 679 (1970).
5. Bodel, P., and Miller, H., Inflammation 1, 103 (1978).
6. Bailey, P. T., Abeles, F. B., Hauer, E. C., and Mapes, C. A., Proc. Soc. Exp. Biol. Med. 153, 419 (1976).
7. Mapes, C., and Sobocinski, P., Amer. J. Physiol. 232, C15 (1977).
8. Pekarek, R., Wannemacher, R., Powanda, M., Abeles F., Mosher, D., Dinterman, R., and Beisel, W., Life Sci. 14, 1765 (1974).
9. Bodel, P., Ralph, P., Wenc, K., and Long, J. C., J. Clin. Invest. 65, 514 (1980).
10. Powanda, M. C., Cockerell, G. L., Moe, J. B., Abeles, F. B., Pekarek, R. S., and Canonico, P. G., Amer. J. Physiol. 229, 479 (1975).
11. Moe, J., B., Canonico, P. G., Stookey, J. L., Powanda, M. C., and Cockerell, G. L., Amer. J. Vet. Res. 36, 1505 (1975).
12. Greisman, S. E., and Hornick, R. B., Proc. Soc. Exp. Biol. Med. 139, 690 (1972).
13. Dinarello, C. A., Renfer, L., and Wolff, S. M., Proc. Nat. Acad. Sci. USA 74, 4624 (1977).
14. Bornstein, D I., Brendenberg, C., and Wood, W. B., Jr., J. Exp. Med. 117, 349 (1963).
15. Crutz, W. J., Fed. Proc. 39, 991 (1980).
16. Atkins, E., and Wood, W. B., Jr., J. Exp. Med. 102, 499 (1955).